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Development of a Reverse Transcription-Polymerase Chain Reaction Assay for Diagnosis of Lymphocytic Choriomeningitis Virus Infection and Its Use in a Prospective Surveillance Study

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Lymphocytic choriomeningitis virus (LCMV), which is one of several arenaviruses that are pathogenic for humans, causes encephalitis and meningitis in man. In this study, single-stage and nested reverse transcription-polymerase chain reaction (RT-PCR) assays were developed that targeted the GPC and N genes of LCMV. Both assays detected <1 $TCID_{50}$ unit of LCMV. These assays were used to measure the incidence of LCMV infection by testing cerebrospinal fluid (CSF) samples with ≥10 leukocytes/μl collected over 1 year from patients undergoing lumbar puncture for diagnostic reasons at two Birmingham hospitals. Samples were tested for the presence of LCMV RNA by using the RT- PCR assay and for LCMV-specific IgM antibody by using an ELISA assay. None of the specimens collected from 813 patients was positive by either assay. Although no cases of acute infection were detected, 4% (11/272) of serum collected from a subset of patients was positive for LCMV-specific IgG. A significantly greater rate of seropositivity was found among subjects over 60 years of age (9.4%; $P < 0.025$) than was found in younger subjects (2.4% at 30–59 years of age; 0% at <30 years of age). These data suggest that serious central nervous system disease due to LCMV infection is not common in this population. The high rate of seropositivity in those over 60 years of age suggest that infection was once more common. ***J Med Virol 51:107–114, 1997.*** © 1997 Wiley-Liss, Inc.

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INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV) is a member of the family *Arenaviridae*. Arenaviruses are enveloped viruses with a segmented, ambisense RNA genome. The small RNA segment (S-RNA) contains the glycoprotein (GP) and nucleocapsid protein (NP) genes in opposite orientations. The GP gene is transcribed as a single protein and is cleaved to form GP1 and GP2, the virion surface glycoproteins. The large RNA (L-RNA) codes for the viral polymerase and for at least one other nonstructural protein (Bishop and Auperin, 1987). LCMV is a member of the Old World group of arenaviruses, which includes Lassa fever virus and two other closely related viruses (Rowe et al., 1970). The New World group of Arenaviruses has at least 11 members, four of which have been associated with human disease. Several of the arenaviruses cause viral hemorrhagic fevers, including Lassa fever, Argentine hemorrhagic fever (Junin virus), Bolivian hemorrhagic fever (Machupo virus), Venezuelan hemorrhagic fever (Guanarito virus), and an incompletely characterized hemorrhagic fever that has been found thus far only in Brazil (Sabia virus). All of the arenaviruses that are known to be pathogenic for humans are transmitted from rodent reservoirs to humans by direct contact with rodent excretions, particularly urine (Salas et al., 1991). The principal reservoir for LCMV is the common house mouse *Mus musculus* (Lehmann-Grube, 1971). Ham-

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sters have also been reservoirs in several outbreaks of human disease (Skinner et al., 1976). The geographic distribution of each arenavirus is determined by the range of its reservoir species. Thus, LCMV is distributed widely due to the nearly world-wide distribution of *Mus musculus*.

In 1960, Meyer et al. reported that 8% (57/713) of aseptic meningitis and encephalitis cases seen in Veteran's Administration hospitals in the United States were caused by LCMV. A more recent hospital-based study (Miklos, 1978) in Hungary found that 8.8% (143/1,630) of meningitis cases were associated with LCMV infection. In both of these studies, diagnosis of infection was based on complement fixation, virus neutralization, and fluorescence antibody assays. More recent hospital-based data are not available, because LCMV diagnostic tests are performed rarely; thus, the current incidence of LCMV-associated disease is uncertain (Jahrling and Peters, 1992).

However, two studies (Childs et al., 1991; Stephensen et al., 1992) have reported that adult urban populations in both Baltimore, Maryland, and Birmingham, Alabama, have a 5% prevalence of serum IgG antibody against LCMV. Childs et al. (1992) also reported that mice trapped from several urban sites in Baltimore showed serologic evidence of infection, with the prevalence of infection being as high as 13.4% in some areas. Recent case reports from Arizona (Barton et al., 1993) and Nebraska (Larsen et al., 1993) have also reemphasized the fact that LCMV does cause severe central nervous system (CNS) disease in humans. These data also indicate that active transmission of LCMV to humans continues to occur in the United States. Thus, we set out to develop a reverse transcription-polymerase chain reaction (RT-PCR) assay for the diagnosis of LCMV infection and to use this assay to determine the incidence of LCMV-associated inflammatory CNS disease in a 1 year surveillance among patients seen at University Hospital and Children's Hospital in Birmingham, Alabama. Samples of cerebrospinal fluid (CSF) that were collected for routine diagnostic reasons were assayed by using RT-PCR. Matched serum (when available) was also collected and assayed for LCMV-specific IgM and IgG antibodies.

MATERIALS AND METHODS

Viruses and Cell Culture

A strain of LCMV was isolated in our laboratory from a case of callitrichid hepatitis (CH) and is referred to as "LCMV-CH" (Stephensen et al., 1995). The Armstrong (ARM) strain of LCMV was obtained from ATCC (ATCC VR-134). LCMV-CH and ARM were cultured in Vero cells with Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Rockville, MD) supplemented with 5% neonatal calf serum (Gibco- BRL).

Subject Recruitment

This study was approved by the Institutional Review Board for Human Use of the University of Alabama at Birmingham. Enrollment at University Hospital began on January 25, 1993, and ended on January 24, 1994. CSF and sera from the Hospitals' clinical laboratories that were collected for routine diagnostic reasons were used for the study. Samples for collection were identified by screening laboratory records 6 days per week to identify all inpatients and outpatients who had undergone a lumbar puncture. Typically, CSF samples were stored at 4°C for several days before being discarded, allowing time for sample identification. Samples were then stored at −85°C until use. There were 968 lumbar punctures made for CSF collection involving 807 patients during the study period. A total of 880 CSF specimens from 729 patients were located and collected. Only CSF specimens that had ≥10 leukocytes per μl or for whom a request for leukocyte counts was not made were eventually chosen for study. A total of 405 specimens from 320 patients met these criteria. Leukocyte counts were not requested on 53 of these specimens. Ten leukocytes per μl is a conservative cut off for identifying persons with an inflammatory response in the CNS, so it is likely that all encephalitis and meningitis cases would be included in this sample. From among the 729 patients from whom CSF samples were available, 312 matched sera were identified (from 272 patients) and were also collected. Of the 968 patients who underwent lumbar puncture, hospital record data were available for 810. Of these, 22% (179) were infants from the neonatal intensive care unit, 20% (164) were elderly patients (≥60 years old), and 52.3% (424) were 20–59 years old. There were only two (0.2%) children (1–9 years old) in this group. Gender was distributed almost equally between 51.6% (417/808) males and 48.4% (391/808) females. At Children's Hospital, CSF specimens from children with ≥10 leukocytes per μl were saved routinely for another research protocol, and we used these specimens. Between April 20, 1993, and April 19, 1994, 495 of these specimens were collected from 408 patients. From among this pool, 351 CSF samples from 301 patients were actually available and were saved for analysis. For logistic reasons, matched sera were not collected at Children's Hospital. Among the 408 Children's Hospital patients, there were 256 infants (62.7%) and 38 adolescents 10–19 years old (9.3%). None of the Children's Hospital patients was over 20 years old. Gender distribution was 55.5% (222/400) males and 44.5% (178/400) females.

RT-PCR Assay Protocol

Three RNA isolation methods were compared for preparation of LCMV genomic RNA for reverse transcription: 1) proteinase K digestion in the presence of 0.5% sodium dodecyl sulfate (SDS) followed by phenol:chloroform extraction (Higuchi, 1989); 2) guanidinium thiocyanate (GUSCN) denaturation followed by phenol:chloroform extraction (Boom et al., 1990); and 3) no extraction. These three methods were performed by using 100 μl of LCMV culture supernatant followed by the RT-PCR protocol described below. The GUSCN extraction method gave the best results, yielding the heaviest band on an ethidium bromide-stained gel (data

not shown). Briefly, a CSF or other sample (10–100 μl) was added to a tube containing silica particles (Sigma Chemical Co., St Louis, MO) and GUSCN containing lysis buffer (50% GUSCN, 0.1 M Tris-HCl, 0.02 M EDTA, 1% Triton X-100, pH 8.0). Cells and viruses were lysed and released RNA bound to silica particles, forming complexes that could be separated easily by centrifugation (×12,000*g* for 15 seconds). These complexes were washed several times with washing buffer (50% GUSCN, 0.1 M Tris-HCl, pH 6.4) followed by 70% ethanol. The complexes were dried, and RNA was subsequently eluted in 40 μl of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE buffer) by heating at 95°C for 5 minutes.

After this extraction, reverse transcription was performed with 4 U avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI) in a 20 μl reaction volume at 42°C for 30 minutes in 1× reaction buffer (supplied by the manufacturer) with 1 U/μl RNasin (Promega), 1 mM dNTP, and 1 mM upstream primer (GPC11 for the GPC gene or NP16 for the NP gene, as described below). The reaction mixtures were heated to 95°C for 5 minutes to inactivate the reverse transcriptase. cDNA was collected for the "hot-start" PCR reactions after centrifugation (×12,000*g* for 2 minutes). The 100 μl PCR reactions were performed as follows: First, 50 μl of 1 × PCR buffer (supplied by the manufacturer) containing 0.5 mM downstream primer (LCMV 11 or NP16), 0.5 mM upstream primer (GPC4, or NP17-1), and 1 mM dNTPs were placed in a 0.5 ml reaction tube with 1 Ampliwax pellet (Perkin Elmer, Branchburg, NJ). Tubes were then incubated at 75°C for 10 minutes to melt the pellet (to form a wax layer) and were chilled on ice. Fifty microliters μl of 1 × PCR buffer containing the first-strand cDNA and 2.5 U *Taq* polymerase (Promega) were then placed on top of the wax layer. Thermoreactions were cycled 40 times with a denaturing temperature of 94°C for 60 seconds, annealing at 50°C for 60 seconds, and extension at 72°C for 60 seconds. The PCR assays were performed on either a Coy Tempcycler (model 50, Coy Laboratory Products Inc., Ann Arbor, MI) or a Perkin-Elmer Cetus DNA Thermocycler (Perkin-Elmer, Norwalk, Connecticut). The PCR products were run on a 4% NuSieve 3:1 agarose gel, stained with ethidium bromide, and visualized by using ultraviolet (UV) transillumination (Sambrook et al., 1989).

All CSF samples were examined by using PCR in experiments containing positive and negative controls for both the reverse transcription step (infectious LCMV) and the PCR step (plasmid DNA). Because of the large number of specimens, five CSF samples (100 μl per sample) were pooled before the extraction step was performed. PCR was then performed on individual samples if pools showed positive results. CSF samples were tested to determine whether they interfered with the nested RT-PCR assay targeting the nucleocapsid gene (using primers NP16 and 17-1). From 20 to 100 μl of virus supernatant containing 200–1,500 $TCID_{50}$ units of LCMV-CH were added to 100 μl of CSF from ten different patients. These samples were processed, and all yielded a band of the expected size and intensity product on ethidium bromide-stained gels.

In Vitro RNA Transcription

To assess the sensitivity of the RT-PCR assay, in vitro transcription was performed by using the pCRII 3.9 Kb plasmid (Invitrogen, San Diego, CA) containing the PCR product amplified with primers NP16 and NP17-1 and using LCMV Armstrong genomic RNA as a template. Briefly, purified plasmids containing the NP gene were linearized with Hind III (Promega) and purified. RNA was transcribed from 1 μg linearized plasmid using T7 RNA polymerase (as recommended by the manufacturer; Boehringer-Mannheim Biochemicals, Indianapolis, IN). The reaction mixture was incubated at 37°C for 2 hours. DNase (0.5 unit/reaction; Boehringer-Mannheim Biochemicals) was added to the reaction to remove the plasmid DNA template. The reaction was stopped by adding 0.02 M EDTA. pH 8.0. The resulting RNA was purified by using a Chroma spin-100 column (Clonetech, Palo Alto, CA). RNA concentration was determined by measuring OD at 260 nm.

Southern Blot

A digoxigenin (DIG)-labeled oligonucleotide probe was used to confirm the identity of PCR products. The probe was made by using a modification of previously described methods for detecting PCR products with labeled probe (Begum et al., 1993; De Leon et al., 1992; Cogan and Bowden, 1991). By using a positive pressure blotter (Stratagene, La Jolla, CA), PCR products were transferred from gels onto nylon membranes (Schleicher and Schuell, Keene, NH). The DNA was then cross-linked to the membrane by UV with a Stratalinker (Stratagene). The oligonucleotide probes were 3′ end labeled with DIG-dUTP by using terminal transferase (Boehringer-Mannheim Biochemicals) and were purified by ethanol precipitation. Conditions of hybridization (time, temperature, salt concentration) to the immobilized target DNA were determined, and detection of positive samples was performed by using the Genius nonradioactive detection kit as described by the manufacturer (Boehringer Mannheim Biochemicals). Membranes were incubated in prehybridization buffer for 1 hour at 68°C in a model 12/24 hybridization oven (Unitherm, Natick, MA) and were hybridized at 55°C overnight by using the recommended hybridization buffer [5× standard saline citrate (SSC), 0.1% lauroylsarcosine, 0.02% SDS, and 1% proprietary blocking reagent]. The DIG-labeled probe was present at a concentration of 5–10 ng per ml of hybridization buffer. The membranes were washed twice in 2× SSC/0.05% SDS at room temperature and then twice in 0.5× SSC/0.05% SDS at 45°C. After these posthybridization washes, membranes were incubated with blocking buffer for 3 hours at room temperature. Detection of PCR product-probe hybrids was performed by incubating for 30 minutes at room temperature with an alkaline phosphatase-conjugated anti-DIG antibody (1:5,000 dilution). After washing twice with washing buffer (0.1 M Tris-

HCl, 0.15 M NaOH, pH 7.5), Lumigen-PPD substrate was added to membranes and incubated for 4 minutes at room temperature. Damp membranes were exposed to XAR5 x-ray film (Eastman Kodak, Rochester, NY) for 30 minutes.

Nested PCR

All PCR products from the first-round PCR (using primers NP16 and 17-1) were amplified again with nested internal primers (NP21-1 and 20-1). To measure the sensitivity of the nested PCR, serial dilutions of synthetic RNA and infectious LCMV cell culture supernatant were used. After the first-round PCR, 1 µl of PCR product was used as the template for the second-round amplification. The second-round amplification was performed as described above for the first round.

ELISA

All serum and CSF samples collected from both hospitals were screened for LCMV-specific IgM and IgG by using ELISA as previously described (Childs et al., 1991). A serum sample was considered positive if the ELISA titer was $\geq$1:400. In CSF, the cut-off was $\geq$1:100.

Virus Isolation

Selected CSF specimens that showed ambiguous results (i.e., possible positive) from the PCR- Southern blot assay were used to isolate LCMV by inoculating into Vero cell lines. These inoculated cultures were screened by an indirect fluorescent antibody assay as has been described for another arenavirus (Wulff and Lange, 1975).

Data Management and Analysis

Demographic information and other laboratory data were also collected for each subject. All of the data were analyzed by using the SAS version 6.04 statistical software package program. The χ-square test for nonparametric variables was used for comparisons of proportions among subgroups. Fisher's exact test was substituted for the χ-square test if the observation number was less than 5. A *P* value less than 0.05 was considered to be statistically significant.

RESULTS

Development of RT-PCR Assay Targeting the GPC Gene

We selected candidate primers (19-20mers) from areas of the GPC gene with at least 90% sequence identity by comparing sequence from LCMV strains Armstrong, WE, and CH. Twelve primers were synthesized and were tested in nine combinations (pairings that yielded products of <100 or >500 bp were not considered). These nine pairs were screened initially by determining their ability to detect 40,000 and 4,000 copies of a plasmid containing the GPC gene of LCMV strain CH in a PCR assay using an annealing temperature of 50°C and 1.5 mM Mg. Products of the appropriate size were visualized on ethidium bromide-stained agarose gels. Only primer pairs GPC4/11 and GPC14/10 (Table I) combined to produced PCR amplification products in this assay. Annealing temperatures of 55 and 60°C were also tested but did not improve the sensitivity of these assays. Altering the Mg concentration in the PCR reaction mix did not improve the sensitivity of the GPC14/10 assay beyond 4,000 plasmid copies, but increasing the Mg concentration to 3.5 mM increased the limit of detection of the GPC4/11 primer pair to approximately 1,250 plasmid copies. When the PCR assay utilizing primers GPC4 and GPC11 was combined with Southern blot detection using an internal DIG- labelled oligonucleotide probe (Table I), the assay could detect approximately 328 copies of plasmid containing the target sequence (Fig. 1). When this assay was used to detect LCMV genomic RNA extracted from cell-culture supernatant, the limit of detection was approximately 0.1 $TCID_{50}$ units of virus.

Development of Nested RT-PCR Assay Targeting the NP Gene

Six primers were selected from the NP gene based on comparisons among sequences from LCMV strains Armstrong and WE (the LCMV-CH NP sequence was not available) and from Lassa fever virus. The Lassa fever virus sequence was included in this comparison to identify the most conserved regions of this gene among Old World arenaviruses. Primers had at least 87% sequence identity to the two LCMV strains. The relative sensitivities of these primer pairs were determined in an RT-PCR assay by using serial dilutions of LCMV-CH culture supernatant. Primer pair NP16/17-1 (Table I) showed the greatest sensitivity (using an annealing temperature of 50°C and 1.5 mM Mg). Again, different Mg concentrations and annealing temperatures were tested, with 2.5 mM Mg and 50°C giving the greatest sensitivity, a limit of detection of 0.1 $TCID_{50}$ units of virus.

Thus, primers NP16 and NP17-1 were selected as the outer primers for the nested RT-PCR assay. Primers NP20-1 and NP21-1 were selected as internal primers, and the reaction conditions were optimized essentially as outlined above. The sensitivity of the nested PCR targeting the NP gene was determined in two ways. First, 100-fold serial dilutions of LCMV culture supernatant were tested by using the nested PCR; the limit of detection was found to be 1×0^{-4} $TCID_{50}$ units. This apparently paradoxical result (the detection of substantially less than one infectious virus particle) may be explained by the presence of viral RNA in the cell-culture supernatant, which is not associated with infectious particles (e.g., noninfectious defective particles or RNA-NP complexes released from damaged cells). The second method for measuring the sensitivity of the assay was to determine the number of synthetic RNA templates that could be detected in a similar serial dilution experiment. The first-round PCR detected 5×10^3 copies of RNA, whereas the nested PCR detected 5–50 copies of RNA (Fig. 2).

TABLE I. Sequences and location on LCMV S-RNA genome of oligonucleotides used as PCR primers and probes.

Primer	Sequences	Location	S-RNA nt no.
	GPC PCR—Outer primers (product size, 499 bp)		
GPC4	CGCACCGGGGATCCTAGGC	5′ noncoding	1–19
GPC11	AT(A/G)CTCATGAGTGT(A/G)TGGTC	GPC gene	499–480
	GPC PCR—Oligonucleotide probe for Southern Blot		
GPC32	TTTGAGGCT(T/C)TGCCTCACATCAT(T/C)GATGAGGT	GPC gene	99–130
	NP PCR—Outer primers (product size, 204 bp)		
NP16	CGCACAGTGGATCCTAGGC	3′ noncoding	3375–3357
NP17-1	GCTGAC(T/C)TCAGA(A/G)AAGTCCAACC	NP gene	3172–3194
	NP Nested PCR—Internal primers (product size, 116 bp)		
NP21-1	CCTAGGCATTTGATTGCGC	3′ noncoding	3363–3345
NP20-1	AGAAG(G/A)(T/C)ACTGGTTGC(A/G)TCCTT	NP gene	3248–3270
	NP PCR—Oligonucleotide probe for Southern Blot		
NP35	GTGGACACAGGCGTTGAGGAGGGAGTTGCAGGGTT	NP gene	3249–3283

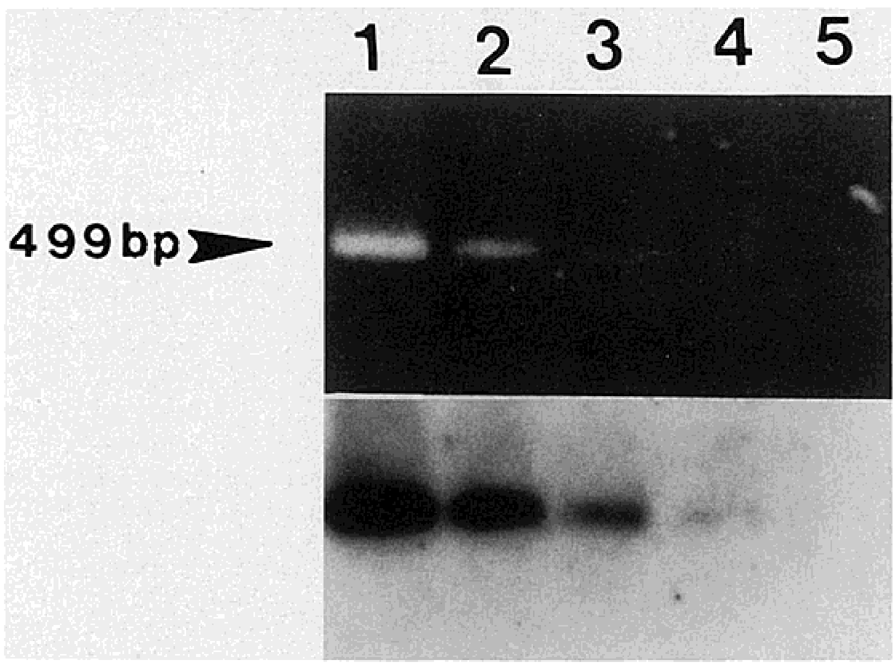

Fig. 1. Southern blotting increases the sensitivity of the polymerase chain reaction (PCR) assay targeting the GPC gene of lymphocytic choriomeningitis virus (LCMV). PCR assays containing 32,800, 3,280, 328, 33, and 0 copies (**lanes 1–5**, respectively) of a plasmid containing the GPC gene of LCMV strain CHV were carried out by using primers GPC4 and GPC11 (reaction Product, 499 bp). Ten microliters of product were then run on a 1.5% agarose gel, stained with 1 μg/ml ethidium bromide, and visualized by using UV transillumination (top). These products were then transferred to nylon membrane and visualized by incubation with a digoxigenin-labelled probe followed by chemiluminescent detection (bottom), as described in the text.

TABLE II. Age distribution of subjects with LCMV-specific IgG serum antibody.

Age groups	Number	Seropositives	Prev. (%)	*p* value
0–29	63	0	0.0	NS[a]
30–59	124	3	2.4	NS[a]
60–	85	8	9.4	$P < 0.025$[b]
Total	272	11	4.0	

[a]By Fisher's exact two-tailed test.
[b]By chi-square test of independence compared to overall prevalence of 4%.

LCMV Detection in CSF Specimens by RT- PCR

Of the 405 CSF samples from University Hospital that met the criteria for screening by RT-PCR (i.e., cell counts ≥10 leukocytes/μl or cell counts not available), 87% contained ≥10 leukocytes/μl. All 405 samples were tested as follows: 1) Supernatants of CSF were tested by using the single-stage RT-PCR assays targeting the GPC and NP genes. 2) Cell pellets were tested by using the single-stage and nested RT-PCR assays targeting the NP gene. Two clinical specimens yielded PCR products of the appropriate size with the GPC assay, and

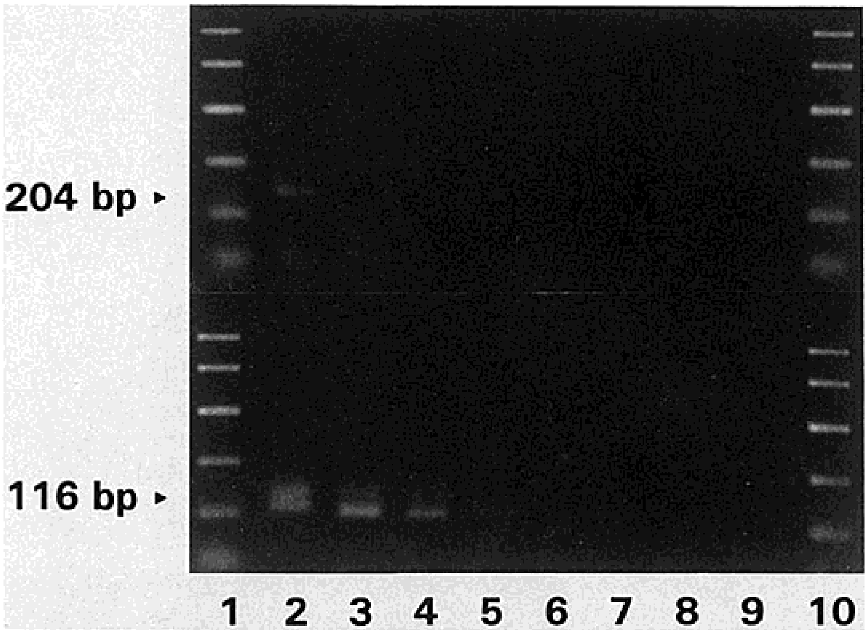

Fig. 2. **Lanes 1–10:** Sensitivity of the reverse transcription (RT)-PCR targeting the nucleocapsid protein (NP) gene of LCMV is increased by second-round amplification with nested primers. The first-round RT-PCR assay was performed by using primers NP16 and NP17-1 (reaction product, 204 bp) on a 100-fold dilution series of tissue culture supernatant containing 10^0, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} $TCID_{50}$ units of LCMV strain Armstrong (lanes 2–8, respectively) or water (lane 9). One microliter of first-round product was then amplified with primers NP21-1 or NP20- 1 (reaction product, 116 bp). Ten microliters of first-round product (top) and second-round product (bottom) were then run on a 1.5% agarose gel, stained with 1 μg/ml ethidium bromide, and visualized by using UV transillumination. Lanes 1–10 contain a 123 bp ladder as a molecular size standard.

one specimen was found with the nested RT-PCR assay targeting the NP gene. One of the specimens was positive with both assays. These PCR products were cloned into plasmids and sequenced to determine whether they were novel isolates or the result of laboratory contamination. Sequence analysis revealed that these products were essentially identical (>99% cDNA sequence identity) to LCMV strain CH, which was used as a positive control during assay of the CSF specimens. The target sequence of the GPC assay showed from 83.8 to 84.6% identity among the three LCMV strains Armstrong, WE, and CH, whereas the target sequence of the NP assay showed 79.6% identity between Armstrong and WE. Thus, these products were false positives attributable to laboratory contamination. The 351 CSF samples from Children's Hospitals (for which cells and supernatant were pooled) were tested by using the single-stage and nested RT-PCR assays targeting the NP gene. None of the Children's Hospital samples were positive by either assay.

LCMV-Specific Antibody Detection in CSF by ELISA

Among the total collection of 880 CSF samples from University Hospital (including the 405 samples tested by RT-PCR) and the 351 CSF samples from Children's Hospital, none was positive for LCMV-specific IgM. There was one CSF specimen from University Hospital that contained LCMV-specific IgG, but it was determined to be a false positive, because this subject also had a high serum IgG titer, and the CSF sample was grossly contaminated with blood. In addition, IgM was not detected in this specimen.

LCMV- Specific Serum Antibody Detection by ELISA

None of the 312 serum samples from 272 patients seen at University Hospital was positive for LCMV-specific IgM. Twelve samples were positive for LCMV-specific IgG from 11 University Hospital patients (4.0%; 11/272). All 11 seropositive subjects were adults >30 years old (Table II). The group over 60 years old showed a significantly greater antibody prevalence than did younger subjects ($P < 0.025$). Similar to other investigators' results (Ackermann, 1973; Ambrosio et al., 1994; Watson et al., 1987), there were no positives in the young adults and children. Most positives were from patients living in Jefferson County (7/11), which includes Birmingham. Of the patients from Jefferson County, 6.7% (7/104) had LCMV-specific serum IgG. Adults >30 years of age from Jefferson County showed a significantly greater prevalence of antibody (8.9%; 7/79; $P < 0.05$) than did younger county residents (0/26). There were no statistically significant differences be-

tween races (Caucasian, 3.6%; African American, 4.8%; $P > 0.5$) or genders (male, 5.0%; female, 2.7%; $P \geq 0.25$).

DISCUSSION

We have developed a sensitive RT-PCR assay for LCMV that can reliably detect <1 $TCID_{50}$ unit of virus and between 5 and 50 copies of a synthetic RNA template. Primers used in this study were designed for detecting a wide range of LCMV strains using all available sequence data. The 3′ end of the LCMV S-RNA segment is highly conserved (Romanowski and Bishop, 1985), with the 19 terminal nucleotides being identical in all arenaviruses (Auperin et al., 1982; Southern and Bishop, 1987). One primer (LCMV 16) was selected from this region. Not surprisingly, this primer was also selected by other investigators for detecting Junin virus (Lozano et al., 1993) and Lassa fever virus (Demby et al., 1994). For these reasons, we feel that the assay will be quite useful for diagnosing LCMV infections.

We have shown that LCMV infection was not detected in association with CNS disease following a year-long surveillance in two Birmingham hospitals. We tested 405 CSF specimens from 320 patients from University Hospital and 351 specimens from 301 patients at Children's Hospital. All were negative by the RT-PCR assay for LCMV. Previous work has demonstrated that LCMV can be isolated from the CSF of patients during the symptomatic phase of infections with CNS manifestations (Lehmann-Grube, 1971). We tested both cell-free supernatants as well as leukocyte pellets, because the results of Ambrosio et al. (1994) indicated that the Junin virus, which is another arenavirus, was isolated successfully only when leukocytes were used. Because of this precaution, the sensitivity of our RT-PCR assay, and its use of primers selected in conserved regions of the LCMV genome, we are confident that the risk of a false-negative test is low. An additional confirmation of our finding no incident cases of LCMV infection by RT-PCR is the fact that all CSF specimens were also negative for LCMV-specific IgM and IgG, and all tested sera were similarly negative for IgM. These confirmatory data are compelling, because elevated levels of LCMV-specific IgM have been found in both serum and CSF samples taken during the acute phase of LCMV- associated CNS disease (Brouqui, 1995; Larsen et al., 1993; Deibel and Schryver, 1976). Elevated IgG titers in CSF have also been found during acute infection (Larsen et al., 1993; Deibel and Schryver, 1976).

Why were no cases of LCMV-associated CNS disease identified when we found a 4% prevalence of serum IgG in the same population? A straightforward explanation could be that most cases of LCMV infection do not result in symptoms that are serious enough to bring infected individuals to the hospital. Previous epidemiologic studies do not support this explanation, however. Clinical data on LCMV infection derives mostly from outbreaks associated with pet hamsters or research animals (Peters, 1991). Some individuals remain healthy (23%), whereas others develop a flu-like illness (48%), but 27% developed meningitis or encephalitis. In one community outbreak that was associated with pet hamsters, 23% (13/57) of infected individuals underwent lumbar punctures due to signs and symptoms of meningitis or encephalitis (Biggar et al., 1975). In a more recent episode in France (Brouqui, 1995), five of six seropositive individuals apparently infected with LCMV from pet hamsters reported no symptoms of infection, whereas the sixth (1/6 or 17%) was admitted to the hospital with typical signs of meningitis. These data indicate that roughly one in four episodes of LCMV infection result in patients seeking medical care at a hospital or physicians office with a work-up for meningitis or encephalitis. Thus, the lack of incident LCMV cases in our series suggests that acute LCMV infection is not common in the area served by these hospitals.

What might account for the apparently low incidence of LCMV infection in this area? We have not examined the prevalence of LCMV infection among mice in Birmingham, but the 4% prevalence of serum IgG antibody against LCMV in our human cohort suggests that LCMV infection of mice does occur. For example, a similar antibody prevalence in Baltimore residents was associated with an overall 9% prevalence of LCMV infection among mice (Childs et al., 1991, 1992), confirming an ongoing risk of human infection. However, the significantly higher prevalence of antibody (9.4%) in those >60 years of age in the present study raises the possibility that human infection is now less common in Birmingham than it has been in years past. The lack of incident cases of LCMV-associated CNS disease is also consistent with this hypothesis. Does this mean that the prevalence of infection in mice has decreased in recent years or that mouse populations have decreased in size, resulting in decreased human contact with infected mice? We cannot rule out these possibilities with our current data, but it seems more likely that improvements in housing or changes in human activity over recent years have resulted in decreased contact between humans and infected mice, thus decreasing the incidence of human LCMV infection in Birmingham. Although this explanation is attractive for its simplicity and plausibility, Childs et al. (1992) found that mouse-human contact is quite common in inner city Baltimore residents, indicating that any postulated decrease in mouse-human contact in Birmingham must be confirmed.

Our finding of no incident cases of LCMV-associated CNS disease during a year-long surveillance is significant and is of public health importance, because the role of LCMV in encephalitis and meningitis has not been examined in a systematic study in the United States since 1960 (Meyer et al., 1960). Because most clinical laboratories do not currently attempt to identify many possible viral pathogens, including LCMV, in patients with suspected viral meningitis or encephalitis, we feel that our development of a sensitive and straightforward procedure for the detection of LCMV will be beneficial for the early and rapid laboratory diagnosis of LCMV infection. It should also be noted that cases

of human LCMV infection continue to appear and that the consequences of such infections can be quite severe, particularly in the case of intrauterine infections (Barton et al., 1993). In addition, the frequent occurrence of LCMV infection in nonhuman primates in many areas of the United States also reminds us that the risk of LCMV infection remains widespread (Ramsay et al., 1989). Thus, it would not be surprising if application of this assay in other areas of the United States reveals a significant incidence of LCMV infection in humans.

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